TEST METHOD PROTOCOL
for the BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test

A Test for Basal Cytotoxicity for an *In Vitro* Validation Study

June 14, 2002

Prepared by

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

Based on Standard Operating Procedure Recommendations from an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health (NIH)
U.S. Public Health Service
Department of Health and Human Services

TEST METHOD PROTOCOL

The BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test
A Test for Basal Cytotoxicity

I. PURPOSE

The purpose of this study is to evaluate the cytotoxicity of test chemicals using the BALB/c 3T3 Neutral Red Uptake (NRU) cytotoxicity test. The data will be used to evaluate the intra- and inter-laboratory reproducibility of the assay and effectiveness of the cytotoxicity assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing the cytotoxicity test and supports the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) and sponsored by NIEHS, U.S. Environmental Protection Agency, and ECVAM. This test method protocol applies to all personnel involved with performing the cytotoxicity assay.

A. Determination of Positive Control Database

An historical database of IC_{50} values for the positive control chemical (Sodium Lauryl [dodecyl] Sulfate {SLS}) must be established and maintained by performing 10 concentration-response assays on the 3T3 cells before performing the NRU assay on test chemicals. Once the mean IC_{50} and the 95 % confidence interval (CI) of the IC_{50} of SLS are established, the values will be used as an acceptance criterion for test sensitivity for the 3T3 NRU assay.

B. BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test

After acceptable positive control mean IC_{50} and 95 % CI values have been established, the 3T3 NRU test will be performed to analyze the *in vitro* toxicity of test chemicals. This test will be used to determine IC_{20}, IC_{50}, and IC_{80} values for a predetermined set of test chemicals of varying toxicities.

II. SPONSOR

A. Name: National Institute of Environmental Health Sciences (NIEHS); The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

B. Address: P.O. Box 12233
Research Triangle Park, NC 27709

C. Representative: *Named Representative*

III. IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals: *Blinded Chemicals*

B. Controls:

Positive:	Sodium Lauryl Sulfate
Vehicle (Negative):	Assay medium

Solvent (as needed): Assay medium with appropriate solvent used to prepare the test chemicals (**Section VII.E**)

IV. TESTING FACILITY AND KEY PERSONNEL

- Name:
- Address:
- Study Director:
- Laboratory Technician(s):
- Scientific Advisor:
- Quality Assurance Director:
- Safety Manager:
- Facility Management:

A. Test Schedule

- Proposed Experimental Initiation Date:
- Proposed Experimental Completion Date:
- Proposed Report Date:

V. TEST SYSTEM

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.

Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

VI. DEFINITIONS

A. *Hill function*: a four parameter logistic mathematical model relating the concentration of test chemical to the response being measured in a sigmoidal shape.

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{logIC50} - X)\text{HillSlope}}}$$

where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logIC50 is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

B. *Documentation*: all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, test chemical preparation, incubator function); all optical density data obtained from the spectrophotometer plate reader will be saved in electronic and paper formats; all calculations of ICx values and other derived data will be in electronic and paper format; all data will be archived.

VII. PROCEDURES

A. Materials

1. Cell Lines

BALB/c 3T3 cells, clone 31

CCL-163, LGC Reference Materials, Customer Service, Queens Road, Teddington, Middlesex, TW110LY, UK

CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA)

2. Technical Equipment

[Note: Suggested brand names/vendors are listed in parentheses. Equivalents may be used.]

a) Incubator: 37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air
b) Laminar flow clean bench/cabinet (standard: "biological hazard")
c) Water bath: 37ºC ± 1ºC
d) Inverse phase contrast microscope
e) Sterile glass tubes with caps (e.g., 5 ml)
f) Centrifuge (optionally: equipped with microtiter plate rotor)
g) Laboratory balance
h) 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ± 10 nm filter
i) Shaker for microtiter plates
j) Cell counter or hemocytometer
k) Pipetting aid
l) Pipettes, pipettors (multi-channel and single channel), dilution block
m) Cryotubes
n) Tissue culture flasks (e.g., 75 - 80 cm^2, 25 cm^2)
o) 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Falcon tissue culture-treated)
p) pH paper (wide and narrow range)

[Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of 3T3 cells.]

3. Chemicals, Media, and Sera

a) Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-332-54)
b) L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)
c) New Born Calf Serum (NBCS or NCS) (e.g., Biochrom # SO 125)
d) 0.05 % Trypsin/0.02 % EDTA solution (e.g., SIGMA T 3924, ICN-Flow, # 16891-49)
e) Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}(for trypsinization)
f) Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}(CMF-HBSS)
g) Dulbecco's Phosphate Buffered Saline (D-PBS) with glucose) formulation containing calcium and magnesium cations, and supplemented with 1000mg/L glucose) (for rinsing)
h) Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)
i) Neutral Red (NR) Dye – tissue culture-grade; liquid form (e.g., SIGMA N 2889); powder form (e.g., SIGMA N 4638)
j) Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (Store under nitrogen @ -20ºC)
k) Ethanol (ETOH), U.S.P. analytical grade (100 %, non-denatured for test chemical preparation; 95 % can be used for the desorb solution)
l) Glacial acetic acid, analytical grade
m) Distilled H_2O or any purified water suitable for cell culture (sterile)
n) Sterile paper towels (for blotting 96-well plates)

[Note: Due to lot variability of NBCS/NCS, first check a lot for growth stimulating properties with 3T3 cells (approximately 20-24 h doubling time) and then reserve a sufficient amount of NBCS/NCS.]

B. Preparations of Media and Solutions

[Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented.]

1. Media

DMEM (buffered with sodium bicarbonate) supplemented with (final concentrations in DMEM are quoted):

a) for freezing (Freeze Medium); contains 2X concentration of NBCS/NCS and DMSO of final freezing solution

40 %	NBCS/NCS
20 %	DMSO

b) for routine culture (Routine Culture Medium)

10 %	NBCS/NCS
4 mM	Glutamine

c) for treatment with Test Chemicals (Treatment Medium)

5 %	NBCS/NCS
4 mM	Glutamine

100 IU	Penicillin
100 µg/ml	Streptomycin

[Note: The serum concentration of treatment medium is reduced to 5 %, since serum proteins may mask the toxicity of the test substance. Serum cannot be totally excluded because cell growth is markedly reduced in its absence.]

Complete media should be kept at approximately 4° C and stored for no longer than two weeks.

2. Neutral Red (NR) Stock Solution

The liquid tissue culture-grade stock NR Solution will be the first choice for performing the assay. If the liquid form is not available, the following formulation can be prepared.

0.4 g NR Dye powder in 100 ml of H_2O

Make up prior to use and store dark at room temperature. May store for up to two months.

3. Neutral Red (NR) Medium

EXAMPLE:

1 ml (4mg NR dye/ml)	NR Stock Solution
79 ml	DMEM

The final concentration of the NR Medium is 50 µg NR dye/ml.
[Note: The NR medium should be incubated overnight at 37ºC ± 1ºC and centrifuged at approximately 600 x g for 10 min (to remove NR crystals) before adding to the cells. Alternative procedures (e.g., Millipore filtering) can be used as long as they guarantee that NR medium is free of crystals.]

4. Ethanol/Acetic Acid Solution (NR Desorb)

1 %	Glacial acetic acid solution
50 %	Ethanol
49 %	H_2O

C. Methods

1. Cell Maintenance and Culture Procedures

BALB/c 3T3 cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 75 - 80 cm^2) at 37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air. The cells should be examined on a daily basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook (see **Section VII.F.3**).

2. Receipt of Cryopreserved BALB/c 3T3 Cells

Upon receipt of cryopreserved BALB/c 3T3 cells, the vial(s) of cells shall be stored in a liquid nitrogen freezer until needed.

3. Thawing Cells

Thaw cells by putting ampules into a water bath at 37°C ± 1ºC. Leave for as brief a time as possible.

a) Resuspend the cells and transfer into Routine Culture Medium in a tissue-culture flask (see **Section 6**).
b) Incubate at 37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air.
c) When the cells have attached to the bottom of the flask (this may take up to 4 h), decant the supernatant and replace with fresh medium. Culture as described above.
d) Passage two to three times before using the cells in a cytotoxicity test.

A fresh batch of frozen cells from the stock lot of cells should be thawed out and cultured approximately every two months. This period resembles a sequence of about 18 passages.

4. Routine Culture of BALB/C 3T3 Cells

When cells exceed 50 % confluence (but less than 80 % confluent) they should be removed from the flask by trypsinization:

a) Decant medium, rinse cultures with 5 ml PBS or Hanks' BSS (without Ca^{2+}, Mg^{2+}) per 25 cm^2 flask (15 ml per 75 cm^2 flask). Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin.
b) Discard the washing solution.
c) Add 1-2 ml trypsin-EDTA solution per 25 cm^2 to the monolayer for a few seconds (e.g., 15-30 seconds).
d) Remove excess trypsin-EDTA solution and incubate the cells at room temperature.
e) After 2-3 minutes (min), lightly tap the flask to detach the cells into a single cell suspension.

5. Cell Counting

After detaching the cells, add 0.1-0.2 ml of Routine Culture Medium/cm^2 to the flask (e.g., 2.5 ml for a 25 cm^2 flask). Disperse the monolayer by gentle trituration. It is important to obtain a single cell suspension for exact counting. Count a sample of the cell suspension obtained using a hemocytometer or cell counter (e.g., Coulter counter).

6. Subculture of Cells

After determination of cell number, the culture can be sub-cultured into other flasks or seeded into 96-well microtiter plates. BALB/c 3T3 cells are routinely passaged at suggested cell densities as listed in the table (approximate doubling time is 20-24 h). The individual laboratories will need to determine and adjust the final density to achieve growth as outlined in **Section VII.C.1**.

Table 1. Cell Densities for Subculturing

Days in Culture	Seeding Density ($cells/cm^2$)	Total Cells per 25 cm^2 flask	Total Cells per 75 cm^2 flask
2	16800	4.2×10^5	1.26×10^6
3	8400	2.1×10^5	6.3×10^5
4	4200	1.05×10^5	3.15×10^5

[Note: It is important that cells have overcome the lag growth phase when they are used for the test.]

7. Freezing Cells

Stocks of BALB/c 3T3 cells can be stored in sterile, freezing tubes in a liquid nitrogen freezer. DMSO is used as a cryoprotective agent.

a) Centrifuge trypsinized cells at approximately 200 x g.
b) Suspend the cells in cold Routine Medium (half the final freezing volume) so a final concentration of $1\text{-}5 \times 10^6$ cells/ml can be attained.
c) Slowly add cold Freeze Medium to the cells so that the solvent will equilibrate across the cell membranes. Bring the cell suspension to the final freezing volume. The final cell suspension will be 10 % DMSO. Aliquot the cell suspension into freezing tubes and fill to 1.8 ml.
d) Place the tubes into an insulated container (e.g., styrofoam trays) and place in a freezer (-70 to -80°C) for 24 h. This gives a freezing rate of approximately 1°C/min. The laboratory needs to ensure that the freezing protocol is applicable to the 3T3 cells and that the cells are viable when removed from cryopreservation.
e) Place the frozen tubes into liquid nitrogen for storage.

8. Preparation of Cells for Assays

a) Cultured cells that are going to be used in seeding the 96-well plates should be fed fresh medium the day before subculturing to the plates. On the day of plate seeding, prepare a cell suspension of 2.5×10^4 cells/ml in Routine Culture Medium. Using a multi-channel pipette, dispense 100 µl Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate (See **Section IV.F**). In the remaining wells, dispense 100 µl of a cell suspension of 2.5×10^4 cells/ml (= 2.5×10^3 cells/well). The seeding density should be noted to ensure that the cells in the control wells are not overgrown after three days (i.e., 24 h incubation in **b** and 48 h exposure to test chemicals). Prepare one plate per chemical to be tested.
b) Incubate cells for 24 h (37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air) so that cells form a less than half confluent monolayer. This incubation period assures cell recovery and adherence and progression to exponential growth phase.
c) Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook.

9. Determination of Doubling Time

a) Establish cells in culture and trypsinize cells as per **Section C.4** for subculture. Resuspend cells in about 5ml Treatment Medium (5 % NBCS/NCS). Seed cells at 4200 cells/cm^2.
b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air).
c) After 4 - 6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue) if Study Director sees a need. Use appropriate size exclusion limits if using a Coulter counter. Determine the total number of cells and document. Repeat sampling at 24 h, 48 h, 72 h, and 96 h post inoculation. Change culture medium at 72 h or sooner in remaining dishes if indicated by pH drop.
d) Plot cell concentration (per ml of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase).

D. Establishing the Positive Control Database

An historical database of IC_{50} values for the positive control chemical (Sodium Lauryl [dodecyl] Sulfate {SLS}) must be established and maintained by performing 10 concentration-response assays on the 3T3 cells.

1. Positive Control Chemical Preparation

The positive control chemical (SLS) is prepared in the same manner as the test chemical (**Sections E.1 and E.2**) by following the instructions and substituting “test chemical” with “SLS.”

2. Range Finder Experiment

Before initiating the 10 concentration-response assays, a range finder experiment will be performed using <u>eight</u> concentrations of SLS by diluting the stock solution with a constant factor as per **Sections E.3.a** and **E.3.b**. The eight chemical concentrations will be tested as per the test procedure outlined in **Section F** and analyzed as per procedures outlined in **Section G**.

3. Test Procedure

Once a range has been determined that satisfies the criteria in **Section E.3.b**, the definitive concentration-response assays shall use a $\sqrt[6]{10} = 1.47$ dilution scheme centered on the IC_{50}. The Test Facility will perform two tests per day on five different days. The 95 % CI of the IC_{50} of SLS will be established and defined as an acceptance criterion for test sensitivity for the 3T3 NRU assay. The confidence intervals shall be calculated using the average of the individual IC_{50} values from each positive control assay performed. An example of an historical mean IC_{50} of SLS in mammalian cultures is **93 µg/ml** and the 95 % CI is **70 - 116 µg/ml** (Spielmann et. al., 1991). All testing will follow the instructions in **Section F** using the 96-well plate configuration in Figure 1. The test meets acceptance criteria if the conditions in **Sections F.5.a.2** and **F.5.a.3** are met.

Figure 1. 96-Well Plate Configuration for Positive Control and Test Chemical Assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
C	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
D	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
E	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
F	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
G	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
H	b	b	b	b	b	b	b	b	b	b	b	b

VC = untreated VEHICLE CONTROL (mean viability set to 100 %)
$C_1 – C_8$ = Test Chemicals or Positive Control (SLS) at eight concentrations (C1 = highest, C8 = lowest)
b = BLANKS (contain **no** cells)

E. Preparation of Test Chemicals

[Note: Test chemical must be freshly prepared immediately prior to use. Each stock dilution should have at least 1-2 ml total volume to ensure adequate solution for the test wells in a single 96-well plate. The solutions must not be cloudy nor have noticeable precipitate. Test chemicals must be at room temperature before dissolving and diluting. Preparation under red or yellow light may be necessary, if rapid photodegradation is likely to occur.]

1. Dissolving Test Chemical

a) Approximately 200,000 µg (200 mg) of the test chemical will be weighed into a glass tube and the weight will be documented. Assay-specific culture medium will be added to the vessel so that the concentration is 2,000,000 µg/ml (2000 mg/ml) and mixed using the mixing procedures outlined in **Section E.1.c**. If complete solubility is achieved, then additional solubility procedures are not needed. The test chemical can then be prepared and diluted for use in an assay. If only partial solubility is achieved, then add additional medium in the steps outlined in Table 1 until the concentration is a minimum of 200,000 µg /ml. If complete solubility at 200,000 µg/ml in culture medium can’t be attained, then repeat the solubility steps in Table 1 using the other solvent(s) in the solubility hierarchy outlined in **Section E.1.c**. Test chemicals that are only soluble in DMSO or ethanol will be prepared at 2,000,000 µg/ml as the highest concentration of stock solution.

Table 1 Determination of Solubility

Solubility Data	Step 1	Step 2	Step 3
Total volume of medium added (ml)	0.1	0.5	1.0
Total volume of DMSO or ethanol added (ml)	0.1	0.5	1.0
Approximate solubility (μg/ml)	≥ 2,000,000	400,000	200,000

Example: If complete solubility is not achieved in 0.1 ml medium (Step 1), then 0.4 ml must be added to obtain a total volume of 0.5 ml (Step 2). No additional weighing of chemical is needed. Chemical and medium are again mixed in an attempt to dissolve.

b) Each test chemical will be prepared such that the highest test concentration applied to the cells in each range finding experiment is 100,000 μg/ml in culture medium (10,000 μg/ml if DMSO or ethanol is used). If 100,000 μg/ml in culture medium cannot be achieved, then the highest concentration attainable will be used. If the range finding experiment shows that 10,000 μg/ml is not high enough for the range of chemicals dissolved in DMSO or ethanol to meet the acceptance criteria, then higher concentrations will be used for the definitive experiment.

c) The following mixing and solvent hierarchy will be followed in dissolving the test chemical:

1) Dissolve test chemical in Treatment Medium.
2) Gently mix. Vortex the tube (1 – 2 minutes).
3) If test chemical hasn't dissolved, use sonication for up to 5 minutes.
4) If sonication doesn't work, then warm solution to 37°C.

If the test chemical doesn't dissolve (i.e., solution is cloudy or has precipitate) in the Treatment Medium, then follow the steps 1) through 4) using DMSO instead of Treatment Medium.

If the test chemical doesn't dissolve in DMSO, then follow steps 1) through 4) using ethanol instead of DMSO.

d) For the range finding experiments, the highest 2x concentration of test chemical dissolved only in culture medium will be 200,000 μg/ml (200 mg/ml). The highest 2x concentration of test chemical first dissolved in DMSO or ethanol then transferred to culture medium will be 20,000 μg/ml (20 mg/ml). Dissolve test chemical in appropriate medium/solvent (at 200-fold the desired final test concentration in the case of DMSO or ethanol solvents, i.e., 20,000μg/ml). The final solvent (DMSO or ethanol) concentration for application to the cells should be kept at a constant level of 0.5 % (v/v) in the vehicle controls and in all of the eight test concentrations. The following example illustrates the preparation of test chemical in solvent and the dilution of dissolved test chemical in medium before application to 3T3 cells.

Example: Preparation of Test Chemical in Solvent Using a Log Dilution Scheme
1) Label eight tubes 1 – 8. Add 0.9 ml solvent (e.g., DMSO or ethanol) to tubes 2 -- 8.
2) Prepare stock solution of 2,000,000 μg test chemical/ml solvent in tube # 1.

3) Add 0.1 ml of 2,000,000 µg/ml dilution from tube #1 to tube #2 to make a 1:10 dilution in solvent (i.e., 200,000 µg/ml).
4) Add 0.1 ml of 200,000 µg/ml dilution from tube #2 to tube #3 to make another 1:10 dilution (i.e., 1:100 dilution from stock solution) in solvent (i.e., 20,000 µg/ml)
5) Continuing making serial 1:10 dilutions in the prepared solvent tubes.
6) Since each concentration is 200 fold greater than the concentration to be tested, dilute 1 part dissolved chemical in each tube with 99 parts of culture medium (e.g., 0.1 ml of test chemical in DMSO + 9.9 ml culture medium) to derive the 8 2x concentrations for application to 3T3 cells. Each test chemical concentration will then contain 1 % v/v solvent. The 3T3 cells will have 0.05 ml Treatment Medium in the wells prior to application of the test chemical. By adding 0.05 ml of the appropriate 2x test chemical concentration to the appropriate wells, the test chemical will be diluted appropriately (e.g., highest concentration in well will be 10,000 µg/ml) in a total of 0.100 ml and the solvent concentration in the wells will be 0.5% v/v.

Check carefully to determine whether the chemical is still dissolved after the transfer from solvent stock solution to medium, and reduce the highest test concentration, if necessary. Document all test chemical preparations in the Study Workbook.

2. pH of Test Chemical Solutions

Measure the pH of the highest concentration of the test chemical in culture medium using pH paper. Document the pH and note the color of the medium for all dilutions. Do not adjust the pH.

3. Concentrations of Test Chemical

a) Range Finder Experiment

Test eight concentrations of the test chemical/PC by diluting the stock solution with a constant factor covering a large range. The initial dilution series shall be log dilutions (e.g., 1:10, 1:100, 1:1000, etc.).

b) Main Experiment

Depending on the slope of the concentration-response curve estimated from the range finder, the dilution/progression factor in the concentration series of the main experiment should be smaller (e.g., $\sqrt[6]{10} = 1.47$; NOTE: this dilution factor will be used for the definitive positive control assays [**Section VII.D.3**]). Cover the relevant concentration range (≥10 % and ≤ 90 % effect) with at least three points of a graded effect, avoiding too many <u>non-cytotoxic</u> and/or <u>100 %-cytotoxic</u> concentrations. Experiments revealing less than three cytotoxic concentrations in the relevant range shall be repeated, where possible, with a smaller dilution factor. (Taking into account pipetting errors, a progression factor of 1.21 is regarded the smallest factor achievable.)

c) Test Chemical Dilutions

- A factor of $\sqrt[2]{10} = 3.16$ could be used for covering a large range: (e.g., 1 ⇒3.16 ⇒10 ⇒31.6 ⇒100 ⇒316 ⇒1000 ⇒3160 µg/ml).

- The simplest geometric concentration series (i.e., constant dilution / progression factor) are dual geometric series (e.g., a factor of 2). These series have the disadvantage of numerical values that permanently change between logs of the series: (e.g., *log0*-2, 4, 8; *log1*- 16, 32, 64; *log2*- 128, 256, 512; *log3*- 1024, 2048,).

- The decimal geometric series, first described by Hackenberg and Bartling (1959) for use in toxicological and pharmacological studies, has the advantage that independent experiments with wide or narrow dose factors can be easily compared because they share identical concentrations. Furthermore, under certain circumstances, experiments can even be merged together:

EXAMPLE:

10						31.6						100
10				21.5				46.4				100
10		14.7		21.5		31.6		46.4		68.1		100
10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100

The dosing factor of 3.16 (= $^2\sqrt{10}$) divides a log into two equidistant steps, a factor of 2.15 (= $^3\sqrt{10}$) divides a decade into three steps. The factor of 1.47 (= 6√10) divides a log into six equidistant steps, and the factor of 1.21 (= $^{12}\sqrt{10}$) divides the log into 12 steps.

For an easier biometrical evaluation of several related concentration response experiments use decimal geometric concentration series rather than dual geometric series. The technical production of decimal geometric concentration series is simple. An example is given for factor 1.47:

Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration, dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).

- Determine which test chemical concentration is closest to the IC50 value (e.g., 50 % cytotoxicity). Use that value as a central concentration and adjust dilutions higher and lower in equal steps for the definitive assay.

F. Test Procedure

1. 96-Well Plate Configuration

The 3T3 NRU assay for test chemicals will use the 96-well plate configuration shown in Figure 1.

2. Application of Test Chemical

a) Two optional methods for rapidly applying the 2X dosing solutions onto the 96-well plates may be utilized. The first method is to add each of the 2X dosing solutions into labeled, sterile reservoirs (e.g., Corning/Costar model 4870 sterile polystyrene 50 mL reagent reservoirs and/or Corning/Transtar model 4878 disposable reservoir liners, 8-channel). The second method utilizes a “dummy” plate (i.e., an empty sterile 96-well plate) prepared to hold the dosing solutions immediately prior to treatment of the test plate (with cells). The test chemical and control dosing solutions should be dispensed into the dummy plate in the same pattern/order as will be applied to the plate containing cells. More volume than needed for the test plate (i.e. greater than 50 µl/well) should be in the wells of the dummy plate. At the time of treatment initiation, a multi-channel micropipettor is used to transfer the 2X dosing solutions, from the reservoirs or dummy plate, to the appropriate wells on the treatment plate (as described in step c. below). These methods will ensure that the dosing solutions can be transferred rapidly to the appropriate wells of the test plate to initiate treatment times and to minimize the range of treatment initiation times across a large number of treatment plates, and to prevent "out of order" dosing. A third option, though not a recommended option, is to transfer test chemical solutions well by well using a single channel pipettor or repeat pipettor. This option will increase the amount of time needed for test chemical application. The use of a repeat pipettor increases the risk of dislodging cells from the culture plate.

b) After 24 h ± 1 h incubation of the cells, remove Routine Culture Medium from the cells by careful inversion of the plate (i.e., "dump") over an appropriate receptacle. Gently blot the plate on a sterile paper towel so that the monolayer is minimally disrupted. Do not use automatic plate washers for this procedure nor vacuum aspiration.
c) Immediately add 50 µl of Treatment Medium to each well. Then add 50 µl Treatment Medium containing either the appropriate concentration of test chemical, the PC, or the VC (see Figure 1 for the plate configuration). The solutions will be transferred from the dummy plate to the test plate by adding the vehicle control first then lowest to highest dose so that the same pipette tips on the eight channel pipettor can be used for the whole plate.

d) Incubate cells for 48 h ± 0.5 h (37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air).

e) **<u>Positive Control</u>**: For each set of test chemical plates used in an assay, a separate plate of positive control concentrations will be set up following the concentration range established in developing the positive control database. This plate will follow the same schedule and procedures as used for the test chemical plates.

3. Microscopic Evaluation

After at least 46h treatment, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record any changes in morphology of the cells due to the cytotoxic effects of the test chemical, but <u>do not use these records for any quantitative measure of cytotoxicity</u>. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of the assay. Use the following Visual Observations Codes in the description of cell culture conditions.

Visual Observations Codes

Note Code	Note Text
1	Normal Cell Morphology
2	Low Level of Cell Toxicity
3	Moderate Level of Cell Toxicity
4	High level of Cell Toxicity
1P	Normal Cell Morphology with Precipitate
2P	Low Level of Cell Toxicity with Precipitate
3P	Moderate Level of Cell Toxicity with Precipitate
4P	High level of Cell Toxicity with Precipitate
5P	Unable to View Cells Due to Precipitate

4. Measurement of NRU

a) Carefully remove (i.e., "dump") the Treatment Medium and rinse the cells very carefully with 250 µl pre-warmed D-PBS. Remove the rinsing solution by gentle tapping. Add 250 µl NR medium (to all wells including the blanks) and incubate (37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air) for 3 h.
b) After incubation, remove the NR medium, and carefully rinse cells with 250 µl D-PBS.
c) Decant and blot D-PBS from the plate. (Optionally: centrifuge the reversed plate.)
d) Add exactly 100 µl NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.
e) Shake microtiter plate rapidly on a microtiter plate shaker for 20 – 45 min to extract NR from the cells and form a homogeneous solution.
f) Measure the absorption (within 60 minutes of adding NR Desorb solution) of the resulting colored solution at 540 nm ± 10 nm in a microtiter plate reader (spectrophotometer), using the blanks as a reference. Save raw data in the Excel format as provided by the Study Management Team.

5. Quality Check of 3T3 NRU Assay

a) *Test Acceptance Criteria*

1) A test meets acceptance criteria, if the IC_{50} for SLS is within the 95 % CI of the historical mean established by the Test Facility (as per **Section D**).

2) A test meets acceptance criteria if the mean OD_{540} of VCs is ≥ 0.3 and ≤ 1.1.

3) A test meets acceptance criteria if the left and the right mean of the VCs do not differ by more than 15 % from the mean of all VCs.

b) Checks for Systematic Cell Seeding Errors

The <u>absolute value</u> of optical density (OD_{540} of NRU) obtained in the untreated vehicle control may indicate whether the 2.5×10^3 cells seeded per well have grown exponentially with normal doubling time during the two days of the assay. If doubling time experiments were performed using the NRU assay, then the historical optical densities observed during the doubling time experiments can be used for comparison to determine exponential growth.

To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row 2) and the right side (row 11 for the test plates) of the 96-well plate. Aberrations in the cell monolayer for the VCs may reflect a volatile and toxic test article present in the assay.

Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent.

c) *Quality Check of Concentration-Response*

The IC_{50} derived from the concentration-response of the test chemicals will be backed by at least three responses ≥ 10 % and ≤ 90 % inhibition of NRU. If this is not the case, and the concentration progression factor can be easily reduced, reject the experiment and repeat it with a smaller progression factor. Numerical scoring of the cells (see **Section F.3**) should be determined and documented in the Study Workbook.

G. Data Analysis

A calculation of cell viability expressed as NRU is made for each concentration of the test chemical by using the mean NRU of the six replicate values (minimum of four acceptable replicate well) per test concentration (blanks will be subtracted). This value is compared with the mean NRU of all VC values (provided VC values have met the VC acceptance criteria). Relative cell viability is then expressed as percent of untreated VC. If achievable, the eight concentrations of each chemical tested will span the range of no effect up to total inhibition of cell viability. Data from the microtiter plate reader shall be transferred to the Excel® spreadsheet provided by the Study Management Team for determining cell viability and performing statistical analyses.

The concentration of a test chemical reflecting a 20 %, 50 %, and 80 % inhibition of cell viability (i.e., the IC_{20}, IC_{50}, and IC_{80}) is determined from the concentration-response by applying a Hill function to the concentration-response data. It will not be necessary for the Testing Facilities to derive the equation since statistical software (e.g., GraphPad PRISM® 3.0) specified by the Study Management Team shall be used to calculate IC_{20}, IC_{50}, and IC_{80} values (and the associated confidence limits) for each test chemical. In addition, the Study Management Team shall provide guidelines for calculating ICx values and confidence limits. The Testing Facility shall report data using at least three (3) significant figures and shall forward the results from each assay to the Study Management Team/biostatistician through the designated contacts in electronic format and hard copy upon completion of testing. The Study Management Team will be directly responsible for the statistical analyses of the Validation Study data.

VIII. REFERENCES

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IX. APPROVAL

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SPONSOR REPRESENTATIVE **DATE**
(Print or type name)

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Test Facility STUDY DIRECTOR **DATE**
(Print or type name)